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1 **Evidence for niche differentiation in the environmental responses of co-occurring**
2 **mucoromycotinian fine root endophytes and glomeromycotinian arbuscular**
3 **mycorrhizal fungi**

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20

21 **Abstract**

22 Fine root endophytes (FRE) were traditionally considered a morphotype of arbuscular
23 mycorrhizal fungi (AMF), but recent genetic studies demonstrate that FRE belong within the
24 subphylum Mucoromycotina, rather than in the subphylum Glomeromycotina with the AMF.
25 These findings prompt enquiry into the fundamental ecology of FRE and AMF. We sampled
26 FRE and AMF in roots of *Trifolium subterraneum* from 58 sites across temperate southern
27 Australia. We investigated the environmental drivers of composition, richness, and
28 colonisation of FRE and AMF by using structural equation modelling and canonical
29 correspondence analyses. Root colonisation by FRE increased with increasing temperature
30 and rainfall; but decreased with increasing phosphorus. Root colonisation by AMF increased
31 with increasing soil organic carbon but decreased with increasing phosphorus. Richness of
32 FRE decreased with increasing temperature and soil pH. Richness of AMF increased with
33 increasing temperature and rainfall, but decreased with increasing soil aluminium and pH.
34 Aluminium, soil pH, and rainfall were, in decreasing order, the strongest drivers of
35 community composition of FRE; they were also important drivers of community composition
36 of AMF, along with temperature, and in decreasing order: rainfall, aluminium, temperature,
37 and soil pH. Thus FRE and AMF showed the same responses to some (e.g., soil P, soil pH)
38 and different responses to other (e.g., temperature) key environmental factors. Overall, our
39 data are evidence for niche differentiation among these co-occurring mycorrhizal associates.

40

41 **Keywords:** Arbuscular mycorrhizal fungi, fungal ecology, Glomeromycotina, *Glomus tenue*,
42 Mucoromycotina, *Trifolium subterraneum*.

43

44 **Abbreviations**

45 Al = aluminium

46 AMF = arbuscular mycorrhizal fungi

47 CCA = canonical correspondence analysis

48 FRE = fine root endophytes

49 K = potassium

50 OC = organic carbon

51 OTU= operational taxonomic unit

52 P = phosphorus

53 SEM = structural equation model

54 S = sulfur

55

56 **Introduction**

57 Arbuscular mycorrhizal fungi (AMF) are widespread symbiotic fungi that form associations
58 with most plants on earth and are found in almost all terrestrial ecosystems (Brundrett 2009).
59 These fungi are characterised by their unique ability to form arbuscules, a finely branched
60 structure formed within plant root cells that facilitates nutrient exchange between plant and
61 fungus (Wang et al. 2017). Fine root endophytes (FRE) were thought to be a morphotype of
62 AMF, characterised by fine hyphae (< 2 µm diameter) and fan-like colonisation patterns
63 (Orchard et al. 2017b; Thippayarugs et al. 1999). However, a recent genetic study has
64 demonstrated that FRE belong within the subphylum Mucoromycotina (order Endogonales)
65 rather than Glomeromycotina, where AMF are placed (Orchard et al. 2017a). This historical
66 misclassification stems from the fact that both fungal groups produce arbuscules and have
67 somewhat similar colonisation morphology; recent work shows that they also have similar
68 intracellular nutrient concentrations and arbuscule senescence patterns (Albornoz et al. 2020).

69 These similarities, particularly the intracellular nutrient concentrations, suggest that
70 arbuscules of FRE function in a comparable manner to those of AMF (Albornoz et al. 2020;
71 Field et al. 2019). The disparate taxonomy, yet probably similar function, prompts our
72 enquiry into the fundamental ecology of mucoromycotinian FRE and glomeromycotinian
73 AMF.

74 Mucoromycotinian FRE form associations with many plant lineages, as do
75 glomeromycotinian AMF (Hoysted et al. 2018; Orchard et al. 2017b). In fact, both are found
76 in the fossil records as far back as the early Devonian period approximately 407 million years
77 ago (Strullu-Derrien et al. 2018), and it has been hypothesised that both FRE and AMF
78 facilitated plants in their colonisation of land (Field and Pressel 2018). Hence, both FRE and
79 AMF have had equal opportunities for evolution and speciation. The diversity of both fungal
80 groups is unknown, but it is estimated that there are over 1,000 operational taxonomic units
81 (OTUs) of AMF (Kivlin et al. 2011; Opik et al. 2014), while there is no accurate estimate for
82 the diversity of FRE. Thippayarugs et al. (1999) identified five morphotypes of FRE, while
83 other studies using DNA sequencing tools, have found between seven (Hoysted et al. 2018)
84 and 42 (Orchard et al. 2016) OTUs of FRE. However, diversity of FRE has been vastly under
85 studied in comparison to AMF. This raises the question as to whether FRE are as diverse as
86 AMF, and if so, what environmental factors might constrain their diversity.

87 In extant plants, FRE often co-occur with AMF, even in the same root segments (Jeffery et al.
88 2018; Orchard et al. 2017c; Ryan and Kirkegaard 2012). For non-vascular plants (e.g.
89 liverworts), Field et al. (2019) proposed that their co-occurrence is due to a complementarity
90 of both fungal groups for plant fitness and, specifically, that each may differ in the nutritional
91 benefits they offer the host. It is an open question whether the AMF and FRE of liverworts
92 have similar ecology, let alone how the ecology of these two groups differs in late-divergent

93 vascular plants. To our knowledge, there has been only a handful of attempts to define the
94 environmental factors that limit the distribution and abundance of FRE in vascular plants, but
95 these studies have focused almost entirely on morphological assessment of colonised roots,
96 neglecting their species diversity (Orchard et al. 2017b).

97 Current evidence suggests FRE are better adapted to extreme environments than AMF, such
98 as waterlogged and acidic soils, and colder climates. For example, Orchard et al. (2016)
99 found higher colonisation by FRE than AMF in a pasture under waterlogged conditions.
100 Global distributional data also suggest FRE are more abundant than AMF in colder climates,
101 such as extreme alpine and polar regions (Bueno de Mesquita et al. 2018; Olsson et al. 2004;
102 Orchard et al. 2017b; Thippayarugs et al. 1999). Low soil pH has seemed to favour FRE, as
103 they tend to replace AMF as pH decreases (Göransson et al. 2008; Postma et al. 2007). AMF
104 are sensitive to high levels of soluble aluminium (Al), which occurs at low pH, while FRE
105 may play an important role in protecting plants against Al toxicity (Göransson et al. 2008;
106 Postma et al. 2007). These findings suggest environmental factors could be important drivers
107 of the species distributions of FRE and AMF, though more field-based studies are needed to
108 investigate the influence of multiple, interacting environmental factors.

109 In this study, we describe the diversity of FRE and AMF associated with a common annual
110 herbaceous legume, *Trifolium subterraneum,* across the temperate climatic zone of southern
111 Australia (Fig. 1). We investigated the diversity and composition of communities of FRE and
112 AMF, and determined the significance of key environmental factors as drivers of distribution
113 and abundance of these two fungal groups. *Trifolium subterraneum* is an ideal host plant for
114 this study. It is an annual, self-regenerating pasture legume of Mediterranean origin widely
115 utilized for dryland farming in temperate regions (Carroni, 1996). It occurs broadly across
116 continental environmental gradients throughout the entire southern half of Australia with

117 some 29 M ha sown (Nichols et al. 2007). Thus, we captured a significant range of soils and

118 climates, while removing effects of host variability. We related patterns of richness and

119 colonisation to variation in soils and environments using structural equation models (SEMs).

120 These models allow the explicit definition and testing of hypothesised causal (versus

121 correlative) relationships among multiple variables in complex ecological data sets such as

122 this one (Grace 2006). We tested the hypothesis that FRE and AMF respond differently to

123 key environmental factors including soil pH, soil nutrient concentrations, temperature and

124 rainfall. Specifically we hypothesised that FRE would be more abundant and diverse in more

125 acidic, colder, and wetter environments, and AMF the reverse, but that both FRE and AMF

126 would be negatively affected by increasing soil nutrientsSpecifically, we tested the

127 hypothesis that FRE and AMF respond differently to key environmental factors including soil

128 pH, soil nutrient concentrations, temperature and rainfall. We complemented these analyses

129 with canonical correspondence analysis (CCA) of community composition. These analyses

130 are the first step towards describing the diversity and ecology of the newly classified FRE.

131

132 **Materials and methods**

133 *Plant sample collection*

134 *Trifolium subterraneum* was sampled from 58 dryland, non-irrigated pastures across

135 temperate southern Australia in the winter of 2014 (Foster et al. 2017; Fig. 1). We conducted

136 all our sampling during winter to remove seasonality as a confounding factor (Kowal et al.

137 2020; Ryan and Ash 1996). Briefly, intact soil cores containing *T. subterraneum* plants were

138 sampled by farmers, packaged in sealed plastic 900 ml containers (30 × 20 × 5 cm; Fig. S1)

139 and shipped (by courier) to The University of Western Australia (Crawley, Western

140 Australia). Samples were kept within their opened plastic containers and grown under

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141 irrigation on benches in a temperature-controlled (~ 25 °C) quarantine glasshouse for two
142 weeks—this period was considered long enough to allow the plants and their root endophytes
143 to recover physiological processes following shipping.

144

145 *Soil sample collection*

146 Adjacent to each *T. subterraneum* sample, a soil sample from the top 10 cm of the pasture
147 was collected and shipped to The University of Western Australia. Soils were air-dried in the
148 glasshouse, sieved to 2 mm, and sent to CSBP Laboratories (Bibra Lake, WA, Australia) for
149 nutrient analyses. Unless specified otherwise, the methods for soil analyses followed those of
150 Rayment and Lyons (2012). Plant-available P and potassium (K) were measured using the
151 Colwell test (Colwell, 1963). Available sulfur (S) was analysed according to Blair et al.
152 (1991), and organic carbon (OC) and exchangeable Al were determined according to Walkley
153 et al. (1934). Soil pH was measured in $CaCl_2$ in a solution ratio of 1:5.

154 For each of the pastures sampled, GPS data were used to extract detailed temperature and
155 rainfall information derived from the Australian Bureau of Meteorology (http://
156 http://www.bom.gov.au). Climatic data obtained were mean annual rainfall (hereafter
157 'rainfall') and monthly mean minimum temperature (hereafter 'temperature).

158

159 *Root colonisation*

160 After two weeks of growth, three plants were carefully removed from each container and
161 gently rinsed with deionised water before roots were separated from shoots and cleared in 1
162 M KOH and stained with ink in vinegar (5% v/v) as described by Vierheilig et al. (1998).

163 Colonisation by FRE and AMF was scored using the line intercept method (McGonigle et al.
164 1990). One hundred intercept points were observed for three replicate plants from each
165 sample and the percentage of root length colonised by AMF and FRE was calculated for each
166 replicate.

167

168 *DNA extraction and sequencing*

169 From each container, two *T. subterraneum* plants were carefully removed and processed.
170 DNA was extracted from approximately 20 mg of fresh root material using the PowerSoil
171 DNA isolation kit (Mo Bio Laboratories, USA). For each DNA sample (116 in total plus a
172 negative control), 15 ng of DNA were used to amplify approximately 260 bp of the 18S
173 rRNA gene using the AMF primer set AMV4.5NF (5'-AAGCTCGTAGTTGAATTTCG-3')
174 and AMDGR (5'-CCCAACTATCCCTATTAATCAT-3') (Sato et al. 2005) with Nextera XT
175 Index Kit v2 adapters (Illumina). The libraries were sequenced using the MiSeq Reagent Kit
176 v3 600-cycle (Illumina). Following sequencing, VSEARCH (Enns et al. 1990) was used to
177 merge paired-end reads and remove low quality bases, chimeras and low abundance
178 sequences (< 10 sequences). After a quality check, sequences were clustered into OTUs at a
179 97% identity threshold. Then, consensus OTUs were queried against the SILVA 18S rRNA
180 database (Quast et al. 2012). Given the unknown diversity of FRE, we took a conservative
181 approach for the classification of OTUs. Any OTU matching an Endogonales (within
182 Mucoromycotina) species was classified as FRE, and any OTU matching a Glomeromycotina
183 species was classified as AMF (> 95% match to reference sequence and > 90% query cover).
184 Seven samples failed to amplify DNA and were removed from the dataset, leaving 109
185 samples. Negative control did not contain any AMF or FRE sequences after quality checks.

186

187 *Phylogenetic analysis*

188 A phylogenetic tree of the OTUs of FRE was produced, which included reference sequences
189 previously classified as FRE (with the accession number) from NCBI GenBank. Sequence
190 alignments were generated using MUSCLE alignment tool with default parameters (Edgar,
191 2004). The evolutionary history was inferred using the Maximum Likelihood method and
192 Kimura 2-parameter model with 1,000 bootstrap replicates (Kumar, 1980). The tree with the
193 highest log likelihood is shown. Initial tree(s) for the heuristic search were obtained
194 automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise
195 distances estimated using the Maximum Composite Likelihood (MCL) approach, and then
196 selecting the topology with superior log likelihood value. A discrete Gamma distribution was
197 used to model evolutionary rate differences among nucleotide sites. There were a total of 210
198 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et
199 al. 2018).

200

201 *Statistical analyses*

202 Amplicon sequencing is highly sensitive to differences in number of sequences among
203 samples (Dickie 2010), so we rarefied the entire dataset to the smallest sequencing depth (i.e.
204 20,464 sequences; Fig. S2). Rarefaction was done to obtain a robust comparison of relative
205 richness among samples. We used the "rrarefy" function in *vegan* with 10 iterations (Oksanen
206 et al. 2017). Then, OTU richness was calculated for each iteration for both FRE and AMF
207 and later averaged. Hereafter, we refer to rarefied richness simply as richness.

208 To test for potential drivers of root colonisation and OTU richness, we built a SEM with soil
209 properties, temperature and rainfall as explanatory variables (Table S1). In the case of OTU

210 richness, root colonisation was also included as an explanatory variable. To build the SEM,
211 we first tested for different combinations of explanatory variables. We included both FRE
212 and AMF as endogenous variables of the same model to allow comparison of the
213 standardised coefficients among the two fungal groups. Models were specified and fitted
214 using the *piecewiseSEM* package (Lefcheck 2016). We compared models for their adequacy
215 via Fisher's C statistic, and the best model was selected.

216 Finally, to test for potential drivers of fungal community composition, we first selected the
217 best explanatory variables for community composition of both FRE and AMF. We included
218 all climatic and soil data (Table S1). Variables with high covariation were inspected and
219 removed using the 'vif' function (Fox and Weisberg, 2019). Then, selection was done with
220 the 'bioenv' function in the *vegan* package (Oksanen et al. 2017). We then used CCA (ter
221 Braak 1986), a constrained ordination technique, to determine the relative importance of each
222 selected environmental variable in explaining patterns of community composition. CCA
223 produces an ordination diagram which shows the patterns of variation in community
224 composition that can be explained best by the environmental variables. A biplot was drawn
225 on the ordination to display the relationship between the explanatory variables and the
226 ordination axes, where the angle and length of the line indicate the direction and strength of
227 the relationship, respectively. All analyses and figures were conducted in R (R Core Team
228 2015).

229

230 **Results**

231 *Diversity of FRE and AMF*

232 We obtained a total of 4,951,873 sequences belonging to 1,115 OTUs. From these, 138,786
233 sequences and 41 OTUs belonged to Mucoromycotina; while 1,371,241 sequences and 164
234 OTUs belonged to Glomeromycotina. The rest of the sequences and OTUs were from other
235 taxa. There was an average of 9.6 OTUs of FRE per root sample, while there was an average
236 of 45 OTUs of AMF per sample. All mucoromycotinian OTUs belonged to Endogonales.
237 Glomerales and Diversisporales were the most abundant glomeromycotinian orders,
238 comprising 68 and 30% of the sequences, respectively. The most abundant OTU of FRE (i.e.
239 OTU_7) comprised 79,051 sequences (i.e. 57% of the total sequences of FRE) and was
240 present in 95% of the samples; while the remaining 59,735 sequences were divided among
241 the other 40 OTUs. The most abundant OTU of AMF (i.e. OTU_1) best matched a reference
242 sequence of *Rhizophagus intraradices*. This OTU comprised 534,153 sequences (39% of the
243 total sequences of AMF), and was found in all samples.

244 Phylogenetic analysis identified three Mucoromycotina clades (proposed herein; Fig. 2). All
245 clades contained OTUs which were associated with colonisation by FRE of *Trifolium*
246 *subterraneum* in the study of Orchard et al. (2017a), together with OTUs identified as
247 endophytes of non-vascular plants (Desiro et al., 2017). Most mucoromycotinian OTUs
248 grouped within Clade A, together with sequences (KX434773.1 and KX434777.1) which
249 dominated *T. subterraneum* roots colonised by FRE in the study of Orchard et al. (2017a)
250 (Fig. 2). Clade A contained the nine most abundant mucoromycotinian OTUs, which
251 comprised more than 90% of the mucoromycotinian sequences identified. Only 10 OTUs
252 grouped within Clade B, together with Endogone *pisiformis* and *E. lactiflua* (Fig. 2); these
253 comprised approximately 4% of the sequences. Only two OTUs grouped in Clade C
254 comprising less than 0.2% of the sequences.

255

256 *Drivers of abundance and richness*

257 Root colonisation by FRE ranged between 0 and 51 % of root length and was present in 74 %
258 of sites (Table S1). Root colonisation by AMF, on the other hand, ranged between 3.2 and 72
259 % of root length and was present at all sites (Table S1). Colonisation by AMF was higher
260 than FRE for all sites, except one (Table S1). Richness of FRE ranged between 1 and 17
261 OTUs, while richness of AMF ranged between 20 and 86 OTUs (Table S1). Richness of
262 AMF was higher than that of FRE for all sites (Table S1).

263 The best SEM was the one containing temperature, rainfall, soil pH, available P, K, S, Al,
264 and OC as explanatory variables for root colonisation, and the same variables plus root
265 colonisation for OTU richness (Table S2S3). However, the effects of K and S were non-
266 significant. Our SEM predicted 31 and 19% of the total variation in root colonisation by FRE
267 and AMF, respectively, and 34 and 50% of the total variation in OTU richness of FRE and
268 AMF, respectively (Fig. 3).

269 Root colonisation by FRE was positively affected by increasing temperature and rainfall; but
270 negatively affected by higher available soil P (Fig. 3a). Rainfall was the strongest driver of
271 root colonisation by FRE (β = 0.37). Root colonisation by AMF was also negatively affected
272 by higher available soil P, but was positively affected by increasing OC (Fig. 3b). Available
273 soil P was the strongest driver of root colonisation by AMF (β = - 0.39).

274 OTU richness of FRE was positively affected by increasing root colonisation by FRE and
275 OC, but negatively affected by increasing temperature and pH (Fig. 3a). Temperature was the
276 strongest driver of richness of FRE (β = -0.34). OTU richness of AMF was positively
277 affected by higher root colonisation by AMF, temperature, and rainfall, but negatively
278 affected by increasing soil pH and available Al (Fig. 3b). Temperature was the strongest
279 driver of richness of AMF (β = 0.45). There was a positive correlation between root

280 colonisation by FRE and AMF ($r = 0.28$, $P < 0.01$; Fig. S2aS3a), but no correlation in
281 richness between FRE and AMF (Fig. S2bS3b).
282
283 *Drivers of community composition*
284 Canonical correspondence analysis explained 13% of the total variation in community
285 composition of FRE ($\chi^2 = 0.94$, $P < 0.02$; Fig. 4a). The first two axes of the CCA explained
286 71 and 18 %, respectively, of the explained variation in community composition of FRE (Fig.
287 4a). Aluminium, soil pH, and rainfall were selected as the best explanatory variables for
288 community composition of FRE (Fig. 4a). For AMF, CCA explained 13% of the total
289 variation in community composition ($\chi^2 = 0.88$, $P < 0.01$; Fig. 4b), and the first two axes of
290 the CCA explained 39 and 23%, of the explained variation in community composition of
291 AMF, respectively (Fig. 4b). Aluminium, soil pH, rainfall, and temperature were selected as
292 explanatory variables for community composition of AMF (Fig. 4b).
293
294 **Discussion**
295 Recent studies have shown evidence for two distinct arbuscule-forming symbioses —FRE
296 and AMF— in roots of vascular plants, which are phylogenetically distinct (Orchard et al.
297 2017a, Hoysted et al. 2018). This finding has triggered interest in elucidating the evolutionary
298 ecology of these newly-defined types of arbuscular mycorrhizal fungi (e.g. Hoysted et al.
299 2018). Here, we found that some environmental factors driving the abundance and diversity
300 of FRE and AMF were shared, such as the negative effect of increasing soil available P and
301 pH on these two fungal groups. However, we also found that some drivers were different and
302 that intriguingly, in one instance, the two fungal groups responded in an opposite manner to

303 the same environmental factor. Indeed, richness of FRE decreased with increasing
304 temperature, while richness of AMF increased. Based on root colonisation, we found no
305 support for the hypothesis that FRE are more abundant than AMF in colder climates, as
306 suggested by Orchard et al. (2017), at least within the limited temperature ranged assessed
307 here across a temperate environment. In fact, we found evidence of the opposite trend, where
308 root colonisation by FRE increased with temperature across southern Australia. We also
309 found no support for the hypothesis that FRE are better adapted to acidic soils than AMF, if
310 we use speciation as a metric of adaptation, as richness of both groups was higher in more
311 acidic soils. Overall, our results suggest that across temperate southern Australia, FRE were
312 more abundant, and AMF more diverse, in the wetter and warmer climates.

313

314 *Diversity of FRE and AMF*

315 We report that sequences matching Mucoromycotina, which we classified as putative FRE,
316 grouped within three clades, all of which contained OTUs associated with colonisation by
317 FRE of vascular plants (*Trifolium subterraneum*; Orchard et al. 2017a), and OTUs which
318 have been described as endophytes of non-vascular plants (Desiro et al, 2017). Most of the
319 mucoromycotinian OTUs and sequences we detected were associated with Clade A and
320 grouped with those highly abundant OTUs which dominated the Mucoromycotina
321 communities of *T. subterraneum* roots colonised by FRE (Orchard et al., 2017a). This clade
322 is recognised as fitting within Densosporaceae (Desiro et al. 2017). Clade B, on the other
323 hand, was comprised of our mucoromycotinian OTUs, putative FRE OTUs (Orchard et al.,
324 2017a), a saprotrophic species (i.e. *Endogone pisiformis*), and an ectomycorrhizal species
325 (i.e. *Endogone lactiflua*). This suggests that Clade A might be a mycorrhizal clade within
326 Mucoromycotina, while the other clades might harbour a wider diversity of functional

327 groups. Similar results were found by Desirò et al. (2017), and they suggested that non-
328 vascular (i.e. liverwort) mycorrhizal-like symbionts belong within the Densosporaceae
329 family. However, Desiro et al. (2017) also showed that many reference sequences belonging
330 to liverwort symbionts were placed across two families within Endogonales (i.e.
331 Endogonaceae and Densosporaceae), along with species currently considered to be non-
332 mycorrhizal. Hence, unlike Glomeromycotina, in which all known species are considered to
333 be AMF, FRE might not be monophyletic within Mucoromycotina, a situation akin to
334 ectomycorrhizal symbioses formed by Basidiomycota and Ascomycota. It is worth noting
335 that many reference OTUs of FRE used in our phylogenetic analysis are 'putative' FRE. This
336 means there is no empirical evidence that species of such sequences indeed form arbuscules
337 or provide benefits to their hosts, as is the situation with most Glomeromycotinian OTUs.
338 Without sequences from single-species isolates, these sequences will remain putative. Care
339 must be taken when interpreting these phylogenetic results. Available sequences of FRE are
340 short reads (~ 200 bp) and this results in difficulty in resolving the phylogeny of FRE within
341 the Mucoromycotina (Desirò et al. 2017). This limitation is shown in our bootstrap values,
342 which ranged greatly, with many nodes showing values less than 50%. Hence, we caution
343 these clades should be taken as a first exploratory step. Future development of
344 Mucoromycotina-specific primers that amplify longer sequences would help elucidate the
345 true placement of FRE within Mucoromycotina, resolving their phylogeny.

346

347 *Ecological drivers of FRE and AMF*

348 Previous studies have hypothesised that FRE are adapted to colder, wetter, and more acidic
349 environments than AMF (Göransson et al. 2008; Orchard et al. 2016; Ormsby et al. 2007;
350 Postma et al. 2007; Walker et al. 2010;). We found that root colonisation by FRE was higher

351 in wetter environments, while soil acidity also had a positive effect on richness of FRE.
352 Additionally, soil pH and rainfall were identified as factors structuring community
353 composition of FRE. Our results, however, also showed that colonisation of FRE was higher
354 for *T. subterraneum* pastures in warmer regions, contradicting the working hypothesis that
355 they are more abundant than AMF in colder climates. However, richness of FRE was lower
356 in warmer climates, suggesting the importance of environmental filtering (Kivlin et al. 2014).
357 The combined results for root colonisation and richness suggests that perhaps a subset of
358 species dominate in warmer climates. Indeed, 13 OTUs of FRE (31.7% of total OTUs) were
359 absent from the sites with the highest temperatures, while no OTU of FRE were absent from
360 sites with the lowest temperatures. Nevertheless, almost half of all OTUs of FRE occurred
361 throughout the whole temperature gradient. This study was restricted by the distribution of *T.*
362 *subterraneum* within agricultural systems and its low temperature range (range studied here
363 was between -1 and 9.9°C). Studies of extreme cold climates (e.g., montane) and desert
364 climates are needed to understand the temperature limits on distribution of FRE. In contrast
365 to FRE, climatic variables did not affect the abundance of AMF. Rather, their root
366 colonisation was mainly driven by available soil P and OC. Richness of AMF, on the other
367 hand, was positively affected by climate (i.e. temperature and rainfall), but negatively
368 affected by soil chemistry (namely, pH and Al), suggesting that AMF, while not more
369 abundant than FRE, are more diverse in wetter and warmer climates. This contrasting
370 response to some environmental drivers by FRE and AMF, provides further evidence that
371 these are two ecologically distinct fungal groups.

372 We conducted all our sampling during in winter for an annual plant in a Mediterranean
373 environment with an autumn-winter-spring growing season. Temporal variation in root
374 colonisation by AMF and FRE has been previously shown (e.g. Bueno de Mesquita et al.
375 2018; Fuchs and Haselwandter 2004; Ryan and Ash 1996). We chose to standardise our

376 sampling to avoid any phenology bias among samples. A recent study on an early-divergent
377 moss (*Lycopodiella inundata*) found that root colonisation by FRE was greater in autumn
378 than in spring and authors attributed this to changes in temperature and rainfall (Kowal et al.
379 2020). This further supports our results that FRE proliferate in wetter environments, but
380 contradicts our results in regards to temperature. This is likely due to the fact the temperature
381 range used by Kowal et al. (2020) was 6 to 11 °C in spring and ~3 to ~16 °C in autumn;
382 while ours was -1 to 9.9 °C. It is possible that colonisation by FRE follows a hump-shaped
383 patter with temperature. Studies encompassing large-scale temperature and rainfall gradients
384 are needed to evaluate whether this is true

385

386 *Future directions*

387 This is the first study to our knowledge that has investigated the factors that determine the
388 distribution of FRE at a continental scale. A large part of the variation in abundance, richness,
389 and composition remained unexplained, which suggests there are other factors that might
390 have an important role in structuring communities of FRE, such as aboveground plant
391 communities. Even though we sampled only *T. subterraneum* roots, pastures are often not
392 monocultures, and *T. subterraneum* cover can vary considerably (Foster, *personal*
393 *observations*). In addition, both SEM and CCA analyses assume linear relationships among
394 variables, yet environmental drivers can have non-linear effects on abundance and diversity
395 of fungi (Dickie and Reich, 2005; McGonigle, 2001). On the other hand, little is known about
396 the communities of FRE in native ecosystems and how they might be structured in biomes
397 other than temperate ones. Future research is needed to assess communities of FRE
398 characteristic of environmentally-variable native ecosystems and how they differ from those
399 in agricultural and heavily disturbed habitats. This research would shine light onto their host

specificity, function, and global distribution. We conclude that both FRE and AMF belong to the functional group known as arbuscular mycorrhizal fungi, while having distinct ecological niches. This ecological distinction between FRE and AMF suggests that each group of fungi play a unique role in symbiosis and broader ecosystem function.

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Declarations

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This research was initiated through a project funded by an Australian Government Postgraduate Award, a Meat and Livestock Australia Postgraduate Scholarship and a Henry Schapper Postgraduate Research Scholarship to SO. The research was subsequently funded by an Australian Research Council (ARC) Discovery grant (DP180103157) and the Natural Environment Research Council NE/S010270/1. MHR was funded by an ARC Future Fellowship (FT140100103). Provision of field samples and associated background data were

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Conflict of interest statement

All authors state they do not have any conflict of interest in the submission of this manuscript.

Availability of data and material

All data is available in Supporting Information and sequences have been submitted to NCBI (accession number PRJNA648786). Data has been made available in Dryad.

Code availability

Code will not be made publicly available.

Author contribution

SO and KJF conducted the sampling, carried out the glasshouse conditioning, and processed samples for analyses. MPY and MJB supplied revised environmental data sets for field pasture samples taken across southern Australia. DBG contributed to DNA sampling and extraction and liaised with GDB and SH who conducted all the molecular and bioinformatics work, and constructed the phylogenetic tree. MHR, RJS, and IAD provided supervision of SO during her Ph.D as well as statistical advice to FEA. FEA analysed the data. FEA, RJS, and MHR wrote the manuscript. All authors contributed to the manuscript by providing feedback and/or written content.

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591 **Figures**

592

593 **Figure 1**. Map of Australia showing the approximate location of the 58 *Trifolium*
594 *subterraneum* pastures. Yellow triangles represent Western Australian pastures (n = 21),
595 green triangles represent South Australia (n = 13), blue triangles represent Victoria (n = 17),
596 and red triangles represent New South Wales (n = 7).

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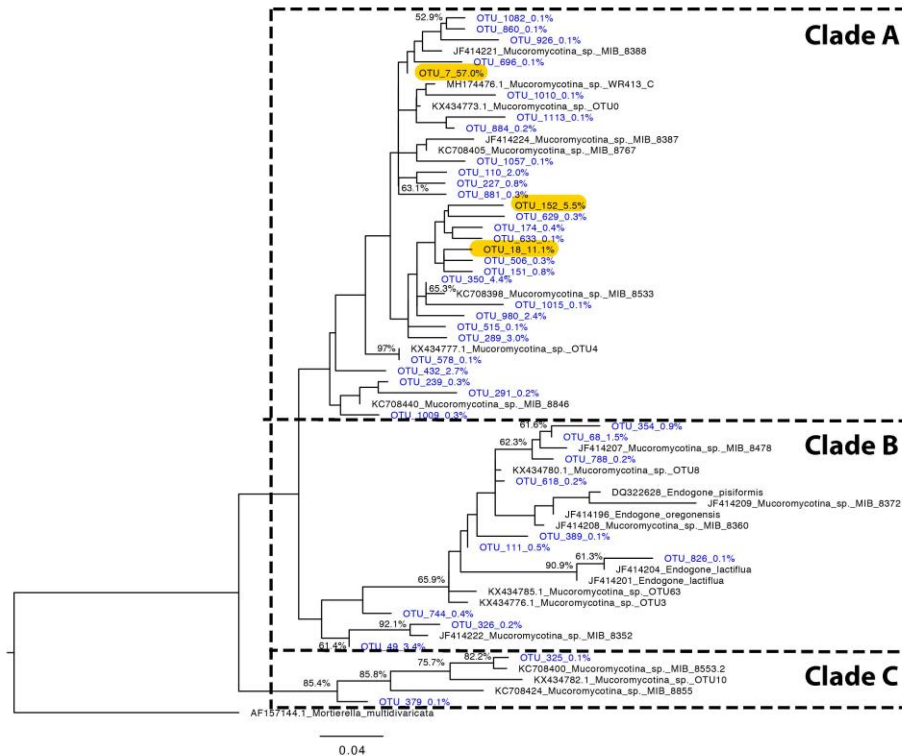
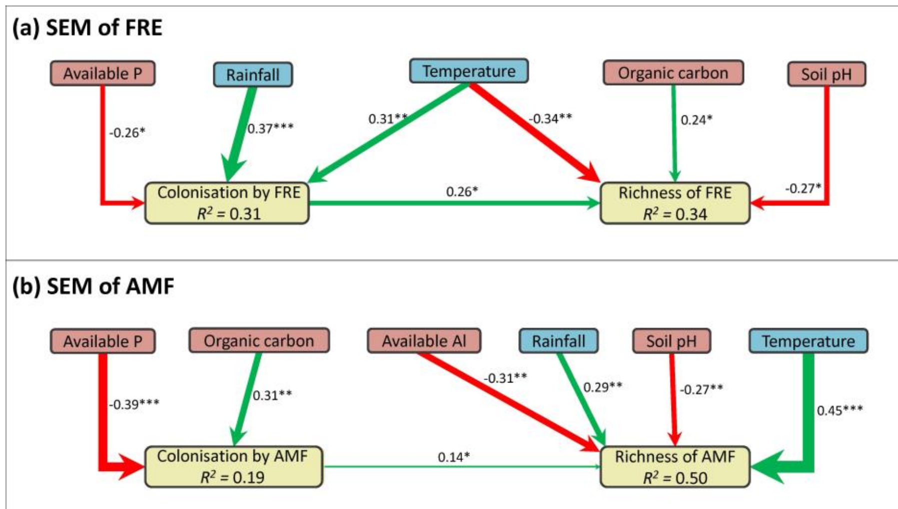


Figure 2. ~~Molecular phylogenetic analysis by a maximum likelihood method~~Maximum likelihood phylogenetic analysis using 18S rRNA gene sequences. Operational taxonomic units (OTUs) coloured blue are from this study and the OTU name includes the percent relative abundance out of all OTU reads for FRE. The three most abundant OTUs are further highlighted in yellow. The branch lengths represent the expected number of substitutions per site. The percentage of bootstrap replicates (out of 1,000) that supported each node (if over 50 %) are shown next to the branches.



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608 **Figure 3.** Summary of structural equation model. For simplicity, only significant effects are
609 shown. The model fitted the data well (χ^2 = 5.66; d.f. = 6; P= 0.46). Explanatory variables
610 were: monthly mean minimum temperature (°C), mean annual rainfall (mm per year), soil pH
611 (in $CaCl_2$), available phosphorus (P; mg kg^{-1}), organic carbon (OC; %), exchangeable
612 aluminium (Al; mg kg^{-1}), and percentage of root length colonised by (a) mucoromycotinian
613 fine root endophytes (FRE) and (b) glomeromycotinian arbuscular mycorrhizal fungi (AMF).
614 The model also included available potassium and sulfur, but they had no effect on fungal
615 variables and hence, for simplicity, are not shown. Green arrows represent a positive effect,
616 while red arrows represent a negative effect. Thickness of arrows is proportional to the
617 standardised coefficient. Total variance explained by the model for each endogenous variable
618 is shown within each box (i.e. R^2).

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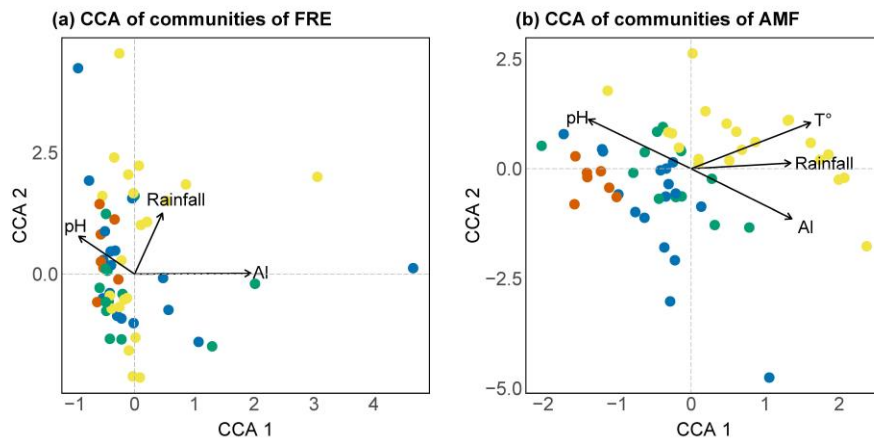

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622 **Figure 4.** Canonical correspondence analysis of the best environmental variables selected
623 with the "bioenv" function in the vegan package. Vector length is proportional to effect size.
624 Al= aluminium (mg kg^{-1}), T° = mean monthly minimum temperature (°C), rainfall = mean
625 annual rainfall (mm), pH = soil pH measured in $CaCl_2$. Colours represent the geographic
626 location (i.e. state) from where samples were obtained: Western Australia (yellow); New
627 South Wales (red); South Australia (green); Victoria (blue) (see Fig. 1).

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